ATTORNEY DOCKET NO. 14014.0349U2
PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	)	
	)	
Blackshear et al.	)	Group Art Unit: 1634
	)	
Application No. 10/049,586	)	Examiner: Sisson, B. L.
	)	
Filing Date: February 12, 2002	)	Confirmation No.: 9700
	)	
For: TTP-RELATED ZINC FINGER DOMAINS AND METHODS OF USE	)	

DECLARATION UNDER 37 C.F.R. § 1.131

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

NEEDLE & ROSENBERG, P.C.
Customer Number 23859

Sir:

We, PERRY J. BLACKSHEAR, ESTER CARBALLO-JANE, and WI S. LAI, hereby declare that:

1. We are the co-inventors named in the above-identified application and co-inventors of the subject matter described and claimed therein.

2. Prior to May 26, 1999, the filing date of U.S. Patent No. 6,627,398, we had conceived and reduced to practice the invention claimed in the above-identified application in the United States or a NAFTA or WTO member country, as evidenced by the attached exhibits.

3. Attached as Exhibit A are relevant pages from an invention disclosure form executed by the inventors prior to the May 26, 1999 filing date of U.S. Patent No. 6,627,398, describing cell and cell-free assays for detecting the binding of TTP to the TTP-binding domain of an AU rich element (ARE) and the scientific bases for such assays.

4. Attached as Exhibit B is a copy of a manuscript by the inventors entitled "Evidence that tristetraprolin binds to AU-rich elements and promotes the deadenylation and destabilization of tumor necrosis factor alpha mRNA." Mol Cell Biol. 1999 Jun;19(6):4311-23. As evident of

the first page of the manuscript, it was submitted prior to the May 26, 1999 filing date of U.S. Patent No. 6,627,398.

5. We declare that all statements made herein of our own knowledge and belief are true and that all statements made on information and belief are believed to be true, and further, that the statements are made with the knowledge that willful false statements are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 3/17/07

PERRY J. BLACKSHEAR

Date: _____

ESTER CARBALLO-JANE

Date: 3-17-2007

WI S. LAI

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Date: _____

PERRY J. BLACKSHEAR

Date: 19 Mar 2007

ESTER CARBALLO-JANE

Date: _____

WI S. LAI

EXHIBIT A

PHS Employee Invention Report	For Patent Branch Use
	E-Number
	U.S.P.A.#
Use plain paper if more space is needed.	U.S. Filing (date)
Part I: To Be Completed by the Inventor	

First Inventor's Name: **Ester Carballo-Jane**

Phone No. [redacted]

This discovery describes Tristetraprolin (TTP), the prototype of a family of cys-cys-cys-his (CCCH) zinc finger proteins, as a factor that binds to and causes the instability of granulocyte-macrophage colony-stimulating factor (GM-CSF) mRNA. The current invention derives from the facts that: 1) The lack of expression of TTP in bone marrow stromal cells (BMSC) derived from TTP deficient mice inhibits the normal degradation of GM-CSF mRNA, resulting in increased levels of this mRNA and increased rates of synthesis and secretion of this growth factor; 2) The absence of TTP in BMSC also inhibits the deadenylation of GM-CSF mRNA, thus decreasing its rate of degradation; 3) Similar phenomena can be observed in transfection studies with constructs expressing TTP and GM-CSF; 4) Similar co-transfection studies have revealed an effect of certain non-binding TTP mutants to *inhibit* the deadenylation and degradation of GM-CSF mRNA, both in cells expressing TTP and in cells not expressing TTP; and 5) Similar phenomena can be observed with the two known mammalian relatives of TTP, TIS-11B and TIS-11D. Although these results are discussed below in relation to TTP specifically, they apply to these related proteins as well.

Our data also provide methods for the development of screening assays for molecules that compete with or otherwise inhibit the action of TTP on GM-CSF mRNA, thus allowing for the increased synthesis of the growth factor. Such screens for TTP competitors or inhibitors are now possible in both intact mammalian or yeast cells, using co-transfection or co-expression approaches, and in cell-free systems, using purified or recombinant TTP or portions of it, the molecules under study (i.e., the potential inhibitors) and RNA oligonucleotides or in vitro synthesized RNAs containing the TTP binding domain of GM-CSF mRNA. Such assays are convenient and are in routine use in our laboratory.

. For the development of gene therapy, various mutant forms of TTP that promote GM-CSF mRNA stability could be screened with these assays, both for absence of direct binding to the GM-CSF mRNA and for their ability to destabilized the mRNA in intact cells. The usefulness of such molecules for gene therapy could be screened in transgenic mouse studies, or in studies with marrow transplantation after marrow transfection with expression vectors. Our future plans involve: 1) Determination of the specificity determinants on both TTP protein and GM-CSF mRNA; 2) Early screens for inhibitory peptides using combinatorial peptide libraries; 3) Development of cell-free assays for TTP-induced deadenylation of GM-CSF mRNA; 4) Study of the effect of TTP on other hemopoietic growth factors that share homology with the GM-CSF ARE; 5)

. Treatments with molecules that compete with or block TTP action on the GM-CSF mRNA, or with modified forms of TTP that are inhibitors of GM-CSF mRNA instability, would be targeted to the mRNA-protein interaction in the cytosol of cells. It is also possible that gene therapy approaches, using modified forms of TTP, would be appropriate for the treatment of some of the conditions listed above.

MOLECULAR AND CELLULAR BIOLOGY, June 1999, p. 4311–4323 Vol. 19, No. 6
0270-7306/99/$04.00+0

Evidence that Tristetraprolin Binds to AU-Rich Elements and Promotes the Deadenylation and Destabilization of Tumor Necrosis Factor Alpha mRNA

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Received 24 February 1999/Accepted 25 March 1999

Mice deficient in tristetraprolin (TTP), the prototype of a family of CCCH zinc finger proteins, develop an inflammatory syndrome mediated by excess tumor necrosis factor alpha (TNF-α). Macrophages derived from these mice oversecrete TNF-α, by a mechanism that involves stabilization of TNF-α mRNA, and TTP can bind directly to the AU-rich element (ARE) in TNF-α mRNA (E. Carballo, W. S. Lai, and P. J. Blackshear, Science 281:1001–1005, 1998). We show here that TTP binding to the TNF-α ARE is dependent upon the integrity of both zinc fingers, since mutation of a single cysteine residue in either zinc finger to arginine severely attenuated the binding of TTP to the TNF-α ARE. In intact cells, TTP at low expression levels promoted a decrease in size of the TNF-α mRNA as well as a decrease in its amount; at higher expression levels, the shift to a smaller TNF-α mRNA size persisted, while the accumulation of this smaller species increased. RNase H experiments indicated that the shift to a smaller size was due to TTP-promoted deadenylation of TNF-α mRNA. This CCCH protein is likely to be important in the deadenylation and degradation of TNF-α mRNA and perhaps other ARE-containing mRNAs, both in normal physiology and in certain pathological conditions.

Tristetraprolin (TTP), also known as Nup475, TIS11, or G0S24 (11, 17, 22, 27, 49), is the product of the immediate-early response genes *Zfp-36* in mouse cells and *ZFP36* in human cells, which map to chromosomes 7 and 19q13.1, respectively (43). TTP is the prototype of a family of zinc finger proteins of the unusual Cys-Cys-Cys-His (CCCH) class; a structure for zinc fingers of this type was recently described, and the finger was shown to bind zinc with high affinity (52). Proteins containing zinc fingers of this class have since been identified in organisms ranging from humans to yeasts (10, 13, 28–30, 35, 40, 43, 47, 50).

TTP is widely distributed, with particularly high levels of expression in spleen, lymph nodes, and thymus (22). Although it was originally described as a nuclear protein in both quiescent and serum-stimulated fibroblasts (11), it was later shown to rapidly translocate from the nucleus to the cytosol upon stimulation with serum or other mitogens (45). This translocation was accompanied by rapid serine phosphorylation of the protein (44). More recently, it has been shown to be almost exclusively cytosolic in macrophage cell lines (45) and in primary mouse macrophages (7).

A link between TTP and the cytokine tumor necrosis factor alpha (TNF-α) was first suggested by studies of TTP-deficient mice (46). These animals appeared normal at birth but then rapidly developed a complex syndrome consisting of dermatitis, alopecia, conjunctivitis, cachexia, myeloid hyperplasia accompanied by extramedullary hematopoiesis, autoimmunity, and erosive polyarticular arthritis (46). Since some aspects of this phenotype resembled earlier mouse models of TNF-α excess (9, 19, 48), we attempted to prevent the development of the TTP deficiency syndrome with weekly injections of anti-TNF-α antibodies. This treatment prevented the development of essentially all aspects of the phenotype (46). Bone marrow transplantation from TTP-deficient mice into RAG-2-immunodeficient mice reproduced the TTP deficiency phenotype after a lag period of several months (6), suggesting that the phenotype might be due to the slow reconstitution of one or more populations of hematopoietic cells. Macrophages from the TTP-deficient mice (derived from bone marrow, fetal liver, or resident peritoneal cells) secreted approximately fivefold-more TNF-α into the medium, accompanied by a twofold increase in cellular levels of TNF-α mRNA, compared to the wild-type macrophages (6). These findings indicated that overexpression of TNF-α from macrophages and perhaps other cells was likely to be important in the development of the TTP deficiency phenotype.

More recently, we showed that the stability of TNF-α mRNA was increased by more than twofold in bone marrow-derived macrophages from TTP-deficient mice compared to cells derived from wild-type mice (half-life = 39 min in the wild type versus 85 min in the TTP-deficient cells) (7). We also demonstrated that both TTP mRNA and protein were induced by both lipopolysaccharide (LPS) and TNF-α itself in normal macrophages (7). These results suggested that TTP might participate in an autoregulatory loop stimulated by TNF-α, so that in the absence of TTP, TNF-α could positively feed back to stimulate and maintain its own expression, resulting in the state of chronic TNF-α excess seen in the TTP-deficient mice (7, 46).

To begin to investigate the apparent ability of TTP to destabilize TNF-α mRNA, we focused on the AU-rich element (ARE) found in the 3′ untranslated region (UTR) of TNF-α from various species (5) as well as in other cytokine mRNAs such as granulocyte-monocyte colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3) (36, 41). These AREs have long been known to confer instability on their respective mRNAs

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(1, 41, 42, 53). We first showed that cotransfection of TTP into HEK (human embryonic kidney) 293 cells with constructs in which the AREs from TNF-α, IL-3, or GM-CSF mRNAs (53) were inserted into the 3′ UTR of β-globin led to the rapid degradation of β-globin mRNA in all three cases (7). We next demonstrated that TTP could bind directly to the TNF-α mRNA, specifically in the ARE region (7). These data indicated that, in some way, TTP could destabilize TNF-α mRNA by binding to its ARE. This was the first demonstration of a direct binding partner for TTP and suggested the possibility that the CCCH family of proteins in general might be RNA binding proteins.

In the present study, we asked whether the integrity of TTP's zinc fingers was necessary for its mRNA-destabilizing and/or direct binding effect and explored the nature of the cleavage of TNF-α mRNA that resulted from TTP binding to its ARE in intact cells. Our data indicate that TTP exhibits zinc finger-dependent ARE-binding activity, as well as a zinc finger-dependent ability to promote TNF-α mRNA deadenylation and degradation. Through regulation of its cellular, subcellular, and tissue-specific expression; induction kinetics; and post-translational modification, this protein offers a myriad of potential mechanisms for regulating the stability of ARE-containing mRNAs.

MATERIALS AND METHODS

Plasmid construction. (i) Parent plasmids. The human TTP (hTTP) cDNA (43) and an hTTP genomic clone were obtained as described elsewhere (23). Plasmid H6E was made by inserting a 3.7-kb *Eco*RI-*Xba*I fragment from the human genomic clone into the plasmid vector pBS+ (Stratagene, La Jolla, Calif.). This insert contained ~1 kb of promoter, the first exon, the single intron, the second exon, and 30 bp of the 3′ flanking region.

(ii) Expression constructs. H6E.HGH3′ was constructed as follows: a 597-bp *Nsi*I-*Xba*I fragment in the 3′ UTR of H6E that contained five rapid degradation signal sequences was replaced by 110 bp of human growth hormone (HGH) sequence that encodes the entire HGH 3′ UTR (GenBank accession no. M13438). The template used to amplify this fragment was pϕGH (Nichols Institute Diagnostics, San Juan, Calif.). The PCR primers were 5′GTGGCTTC TAGatgcatGGGTGGCATC3′ (5′) and 5′GAAGGACACCtctagaGACAAAAT GATGC3′ (3′), where the capital letters correspond to the HGH sequences and the lowercase letters correspond to the recognition sites for *Nsi*I (5′ primer) and *Xba*I (3′ primer).

Construct CMV.hTTP.tag was made as follows. The epitope tag derived from the influenza virus hemagglutinin protein (21) was attached to the last amino acid of hTTP cDNA by the PCR primer-overlapping mutagenesis technique (24). The fusion insert that contained the entire hTTP protein coding region and the hemagglutinin epitope (hTTP.tag) was then cloned into the *Hin*dIII site of the vector CMV.BGH3′/pBS+. The vector CMV.BGH3′/pBS+ was created by blunt end ligating an *Nru*I-*Pvu*II fragment from pRc/CMV2 (Invitrogen, Carlsbad, Calif.), which contains the human cytomegalovirus (hCMV) promoter-enhancer and bovine growth hormone polyadenylation signal, into the *Eco*RI and *Hin*dIII sites of pBS+ (Stratagene). Expression of the fusion protein was confirmed by Western blot analysis of cytosolic extracts from HEK 293 cells transfected with the construct CMV.hTTP.tag, with the polyclonal antibody HA.11 (BAbCO, Richmond, Calif.), which recognized the tag. The zinc finger mutants C124R and C147R of CMV.hTTP.tag, which contained a single amino acid mutation at position 124 or 147, were made by the PCR primer-overlapping mutagenesis technique. In these mutants, the third cysteine in the CCCH motif (C124) of the first zinc finger or the first cysteine (C147) in the second zinc finger was changed to arginine. Mutant S228A of CMV.hTTP.tag, in which the serine at position 228 (equivalent to the mitogen-activated protein [MAP] kinase phosphorylation site S220 in mouse TTP [mTTP] [44]), was mutated to alanine by the same technique. All mutations were confirmed by dideoxy sequencing (Amersham/U.S. Biochemical).

CMV.hTTP.EGFP was made as follows. By the PCR primer-overlapping mutagenesis technique, an *Age*I site was created immediately after the last amino acid of hTTP, so that the stop codon of hTTP was eliminated. When the *Asp*718-*Age*I fragment containing the entire hTTP coding region was inserted into the corresponding restriction sites of plasmid EGFP-N1 (Clontech, Palo Alto, Calif.), hTTP was fused to the N terminus of modified green fluorescent protein (EGFP) in the same reading frame. The zinc finger mutants of CMV .hTTP.EGFP (C124R and C147R) were made by inserting *Bst*EII-*Bam*HI fragments of hTTP containing the mutations from the C124R and C147R mutants of CMV.hTTP.tag into the corresponding restriction sites in CMV.hTTP.EGFP. To make the construct H6E.EGFP, a promoterless fusion construct was created by first removing the CMV promoter from plasmid CMV.hTTP.EGFP by digestion with *Ase*I and *Bgl*II and then blunt end religating the remaining DNA. The hTTP-EGFP fusion plasmid without the promoter was then digested with *Eco*RI (a site in the multiple cloning site of the vector) and *Bst*EII (a site in the hTTP coding region), and then an *Eco*RI-*Bst*EII fragment from plasmid H6E containing ~1 kb of promoter, the first exon, the intron, and part of the second exon up to the *Bst*EII site was inserted into the corresponding sites in the fusion construct.

CMV.mTNF-α was made by first inserting an *Nar*I-*Xba*I fragment containing bp 117 to 1325 of an mTNF-α cDNA sequence (GenBank accession no. X02611) into the *Hin*dIII (blunt end ligation) and *Xba*I sites of vector pSK− (Stratagene); an *Ase*I-*Xho*I fragment containing the hCMV promoter-enhancer from pEGFP-N1 (Clontech) was then blunt end ligated into the *Xho*I site of the vector. Correct orientation of the promoter with respect to the mTNF-α insert was confirmed by dideoxy sequencing. The mTNF-α cDNA clone, provided by B. Beutler (The University of Texas Southwestern Medical Center, Dallas), contained an incomplete 3′ UTR that ended at bp 1325 (GenBank accession no. X02611), with 33 adenylate residues attached to the last T.

CMV.mTNFα (dARE) was made by deleting the ARE region (bp 1302 to 1325 of GenBank accession no. X02611) of CMV.mTNF-α by the PCR primer-overlapping mutagenesis technique. There were 28 adenylate residues attached to the last nucleotide (bp 1301 of GenBank accession no. X02611) of this construct.

Transfection of HEK 293 cells, Northern blot analysis, RNase H assay, and cytosolic extract preparation. HEK 293 cells were maintained in minimal essential medium (Life Technologies, Inc., Gaithersburg, Md.) supplemented with 10% fetal bovine serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Transient transfection of 2×10^6 cells with CMV.mTNF-α or other constructs in calcium-phosphate precipitates was performed as described previously (23, 24), except that the transfection mixture was allowed to stay on the cells for 16 to 20 h and the glycerol shock step was omitted. In some experiments, pXGH5 (Nichols Institute Diagnostics) was also cotransfected to monitor transfection efficiency. Assays of released HGH were performed as described previously (23, 24).

Twenty-four hours after the removal of the transfection mixture, total cellular RNA was harvested from the HEK 293 cells by using the RNeasy system (Qiagen, Valencia, Calif.). Northern blots were prepared as described elsewhere (22). Blots were hybridized to a randomly primed, α-^{32}P-labeled mTTP cDNA (22) or a ~1-kb *Nar*I-*Bgl*II fragment of mTNF-α cDNA. Some blots were also hybridized to an α-^{32}P-labeled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe (7) or a ~0.3-kb fragment of mouse cyclophilin cDNA (bp 166 to 480; GenBank accession no. X52803).

RNase H assays were performed by annealing RNA and oligonucleotide in 10 μl of 50 mM KCl for 5 min at 50°C followed by an additional 10 min at 22°C. The mixture was incubated further at 37°C for 30 min in a buffer (4 mM HEPES-KOH [pH 8], 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM dithiothreitol, and 1 μg of bovine serum albumin per ml) containing 0.8 U of RNase H (Promega, Madison, Wis.), in a final volume of 25 μl. The reaction mixture was then precipitated with sodium acetate and ethanol, and the resulting RNA was subjected to Northern blot analysis.

Cytosolic extracts were prepared from HEK 293 cells 24 h after the removal of the transfection mixture. The cells were incubated on ice for 20 min in a buffer consisting of 10 mM HEPES (pH 7.6), 3 mM $MgCl_2$, 40 mM KCl, 5% (vol/vol) glycerol, 0.5% (vol/vol) Nonidet P-40, 2 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 8 μg of leupeptin per ml (lysis buffer). Lysis of the cells and maintenance of intact nuclei were carefully monitored by microscopy. The nuclei and cell membrane debris were removed by centrifugation at 16,000 × *g* at 4°C for 15 min. Glycerol was added to the supernatant (cytosolic extract) to 20% (vol/vol), and the resulting extract was stored at −70°C.

Analysis of RNA-protein complexes by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), electrophoretic mobility shift assay, and immunoprecipitation. (i) Preparation of RNA probes. Plasmid p3′mTNF-α containing the mTNF-α 3′ UTR (bp 1110 to 1627 of GenBank accession no. X02611) was constructed by reverse transcription-PCR with RNA from RAW 264.7 cells treated for 4 h with 1 μg of LPS (Sigma, St. Louis, Mo.) per ml as a template for reverse transcription. The 5′ primer for PCR amplification was 5′CTTTCCgaattcACTGGAGCCTC3′, and the 3′ primer was 5′TAGAtctagaA GCGATCTTTATTTCTCTC3′, where the lowercase letters indicate the restriction sites for *Eco*RI and *Xba*I, respectively. The resulting PCR product was digested with these enzymes and cloned into the *Eco*RI and *Xba*I sites of the vector pSK− (Stratagene).

Plasmid pTNF-α 1197–1350, which contained a 153-bp fragment containing the ARE of mTNF-α 3′ UTR (bp 1197 to 1350 of GenBank accession no. X02611), was made by PCR with plasmid p3′mTNF-α as a template, with a 5′ primer, 5′GATAagatctCAGGCCTTCC3′, and a 3′ primer, 5′GCCTtctagaTAA ATACATTCATAAGC3′. The resulting PCR product was digested with *Bgl*II and *Xba*I (sites indicated by lowercase letters in the primers) and cloned into the *Bam*HI and *Xba*I sites of the vector pSK−.

Plasmid pTNF-α 1197–1300 (bp 1197 to 1300 of GenBank accession no. X02611), containing only one AUUUA motif, was made with the TNF-α 3′ UTR as template, with the M13-20 primer as the 5′ primer, and a 3′ primer, 5′CTG AtctagaAGTGCAAATATAAATAGAGG3′. The resulting PCR product was

digested with *Eco*RV and *Xba*I (site indicated by lowercase letters in the 3′ primer) and cloned into the corresponding sites of the vector pSK−.

Plasmid pTNF-α 1281–1350 (bp 1281 to 1350 of GenBank accession no. X02611) contained seven AUUUA motifs, five of them being overlapping UUA UUUAUU nanomers. This was constructed by using the TNF-α 3′ UTR as template, with a 5′ primer, 5′GACTggatccTCTATTTATATTTGCAC3′, and the M13 reverse primer as the 3′ primer. The resulting PCR product was digested with *Bam*HI (site indicated by lowercase letters in the 5′ primer) and *Xba*I and cloned into the corresponding sites of the vector pSK−.

Plasmid pTNF-α 1309–1332 (bp 1309 to 1332 of GenBank accession no. X02611), containing four overlapping UUAUUUAUU nanomers, was constructed by inserting double-stranded oligonucleotides spanning bp 1309 to 1332 into the *Eco*RV-*Xba*I cloning sites of pSK−. Plasmid pTNF-α 1309–1332 (A/G), containing the same sequence except that the five A's in the AUUUA motifs were replaced with G's (see Fig. 1), was made by the same technique.

Plasmid pTNF-α 1110–1325 (bp 1110 to 1325 of GenBank accession no. X02611) was made by inserting the *Eco*RI-*Xba*I fragment of the mTNF-α clone from B. Beutler into the corresponding sites of pSK−. This 248-base fragment contained five AUUUA motifs, three of them being clustered nanomers. There were 33 adenylate residues at its 3′ end.

Correct sequences of all plasmid inserts were confirmed by dideoxy sequencing.

To label RNA transcripts with [α-^{32}P]UTP (800 Ci/mmol), the above plasmids linearized with *Xba*I were used as templates, and the Promega riboprobe in vitro transcription system protocol was employed. The resulting product was precipitated with ammonium acetate and ethanol.

(ii) Cross-linking of proteins to RNA. Cytosolic extracts prepared from HEK 293 cells transfected with CMV.hTTP.tag or vector (20 μg of protein) were incubated with 2×10^6 cpm of RNA probe in a 96-well plate at room temperature for 20 min in 20 μl of lysis buffer (without protease inhibitors). Heparin and yeast tRNA were added to final concentrations of 0.5 μg/μl and 50 ng/μl, respectively, for an additional 10 min. The 96-well plate was then placed on ice and irradiated with 254-nm UV light in a Stratalinker (Stratagene) for 30 min at a distance of 5 cm from the light source. RNA not associated with protein was digested with 100 U of RNase T_1 (Life Technologies, Inc.) for 20 min at room temperature and further digested with 1 μg of RNase A (Pharmacia Biotech, Piscataway, N.J.) per μl at 37°C for 15 min. The RNase-resistant RNA-protein complexes were analyzed by SDS-PAGE (10% acrylamide gel) followed by autoradiography.

Identical samples were diluted to 0.5 ml in radioimmunoprecipitation assay buffer, precleared with nonimmune rabbit serum (1:100 dilution; 1 h at 4°C) and protein A-Sepharose (Pharmacia Biotech) (1 h at 4°C), and then incubated overnight at 4°C in the presence of either nonimmune serum (1:100) or a 1:100 dilution of a polyclonal antiserum. Immune complexes were recovered by centrifugation after the addition of protein A-Sepharose, washed three times with wash buffer (50 mM Tris-HCl [pH 8.3], 150 mM NaCl, 1 mM EDTA, 0.5% [vol/vol] Nonidet P-40), resuspended in 100 μl of SDS sample buffer, and subjected to SDS-PAGE on 10% acrylamide gels and autoradiography.

(iii) Western blotting. Cell extracts (5 to 50 μg of protein) were mixed with a 1/5 volume of 5× SDS sample buffer (2), boiled for 5 min, and then loaded onto SDS–10% PAGE gels. Western blotting was performed by standard techniques. Membranes were incubated in Tris-buffered saline–0.5% Tween 20 with either polyclonal antiserum HA.11 (1:2,500); a rabbit antiserum to mTTP, 2640 (1:100 [38]); or a rabbit antiserum to hTTP, DU88 (1:100 [32]). Incubation of the membranes with secondary antibody and development were performed as described elsewhere (6).

(iv) RNA electrophoretic mobility shift assay. Cytosolic extracts prepared from HEK 293 cells transfected with either vector alone, H6E.HGH3′, or expression constructs driven by the CMV promoter (10 μg of protein) were incubated with 10^5 cpm of RNA probe at room temperature for 20 min in 20 μl of lysis buffer (without protease inhibitors). Heparin and yeast tRNA were added to final concentrations of 0.5 μg/μl and 50 ng/μl, respectively, for an additional 10 min. RNA not associated with protein was digested with 100 U of RNase T_1 for 20 min at room temperature; the reaction mixture was then loaded onto a 6% nondenaturing acrylamide gel and subjected to electrophoresis at 250 V for 90 min, in 0.4× Tris-borate-EDTA buffer.

Green fluorescent protein (GFP) assays. Cells were plated onto 100-mm-diameter dishes and transfected with hTTP-EGFP fusion constructs as described above. Twenty four hours after the removal of the transfection mixture, the cells were transferred into four-well Titertek slides (Fisher Scientific, Pittsburgh, Pa.) and incubated at 37°C overnight. The cells were washed once in phosphate-buffered saline, fixed with 3.7% (vol/vol) formaldehyde for 5 min, and washed again with phosphate-buffered saline. Glass coverslips were mounted with Vectashield fluorescent mounting medium (Vector Laboratories, Burlingame, Calif.) and sealed with nail polish. Fluorescence microscopy was performed with a Zeiss confocal microscope model LSM 410 UV (Carl Zeiss, Inc., Thornwood, N.Y.). Images were collected under 488-nm excitation with a 515- to 565-nm emission filter and a 100 × 1.4 numerical aperture oil immersion lens. Photographs were taken with a 16.1-s scan.

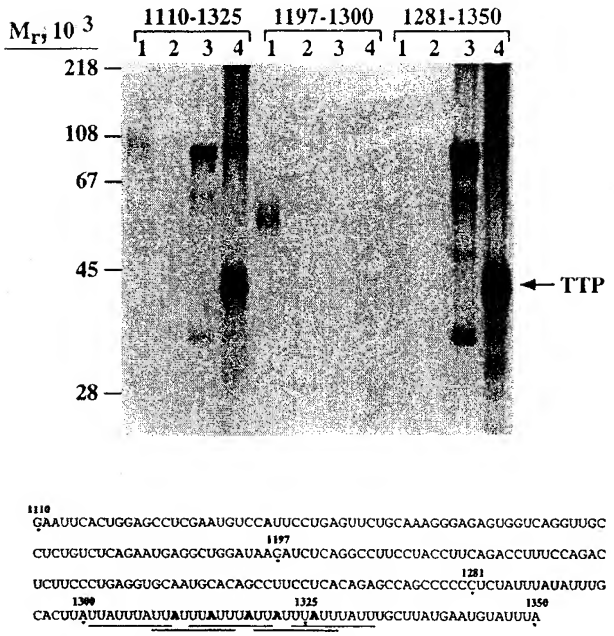


FIG. 1. UV cross-linking of hTTP to TNF-α mRNA ARE probes. Cytosolic extracts were prepared from 293 cells transfected with 5 μg of either CMV .hTTP.tag or vector alone as described in Materials and Methods. Extract (20 μg of protein) was incubated with the indicated ^{32}P-labeled TNF-α RNA probes (2×10^6 cpm). The numbers at the top of each set refer to the base numbers in the mTNF-α mRNA, as shown at the bottom of the figure. Probe 1110–1325 contained approximately 35% U residues, probe 1197–1300 contained 40% U residues, and probe 1281–1350 contained 62% U residues. Heparin and yeast tRNA were then added to decrease nonspecific binding. After UV cross-linking of the probes to cellular proteins, RNases T_1 and A were added to digest probe not cross-linked to protein. The RNase-resistant RNA-protein complexes were resolved by SDS–10% PAGE followed by autoradiography. Lanes 1, probe alone (5,000 cpm); lanes 2, probe (2×10^6 cpm) treated with RNases T_1 and A; lanes 3, extract (20 μg of protein) from 293 cells transfected with vector alone (5 μg of DNA); lanes 4, extract (20 μg of protein) from 293 cells transfected with CMV.hTTP.tag (5 μg). The position of TTP cross-linked to ^{32}P-labeled RNA is indicated by the arrow. The positions of protein molecular weight standards are indicated on the left. Shown at the bottom is a portion of the mTNF-α mRNA 3′ UTR (GenBank accession no. X02611), from which the probes were derived. The five AU-rich nanomers are underlined. The five flanking A's within the ARE that were mutated to form a nonbinding probe are indicated in boldface.

RESULTS

Effect of TTP on TNF-α mRNA species. In most of the expression studies in 293 cells described below, we used a TNF-α expression construct, CMV.mTNFα, that did not contain the entire native 3′ UTR; instead, the TNF-α sequence ended in the middle of the fourth AUUUA motif within the ARE (bp 1325 of GenBank accession no. X02611) (Fig. 1) and was immediately followed by 33 adenylate residues encoded by the vector. To test whether this shortened ARE exhibited TTP binding activity, we compared TTP binding to a 3′-truncated RNA probe, comprising bases 1110 to 1325 of GenBank accession no. X02611, to its binding to a nontruncated probe, comprising bases 1281 to 1350. This nontruncated probe contained the seven natural AUUUA motifs, five of them in clustered nanomers (Fig. 1). We recently demonstrated that TTP could bind directly to probe 1197–1350 probe (7). UV cross-linking of these probes to proteins in extracts from CMV .hTTP.tag-transfected cells indicated that TTP bound to the truncated probe 1110–1325 almost as well as to the probe containing all of the native AUUUA motifs (probe 1281–1350) (Fig. 1). A probe spanning bases 1197 to 1300, which contained

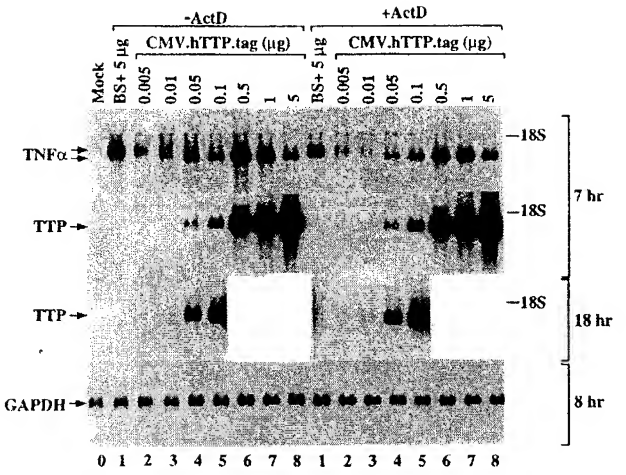


FIG. 2. Effect of TTP on TNF-α mRNA stability. CMV.mTNF-α was cotransfected into 293 cells with either TTP expression constructs or vector alone. After addition of actinomycin D to a final concentration of 10 μg/ml (+ActD) or buffer alone (−ActD) for 4 h, total cellular RNA was harvested. Each lane was loaded with 10 μg of total RNA. Electrophoresis and Northern blot hybridization were performed as described in Materials and Methods. Lane 0, RNA from mock-transfected 293 cells. Lanes 1 to 8, RNA from 293 cells cotransfected with CMV.mTNF-α (1 μg) and vector or CMV.hTTP.tag as follows: lanes 1, vector alone (BS+; 5 μg/plate); lanes 2 to 8, CMV.hTTP.tag (0.005, 0.01, 0.05, 0.1, 0.5, 1, and 5 μg/plate, respectively). Vector was also added in lanes 2 to 7 to make the total amount of cotransfected plasmids 5 μg/plate. The Northern blots were probed with either a ^{32}P-labeled mTNF-α cDNA or a ^{32}P-labeled mTTP cDNA. The two arrows indicate the two species of TNF-α mRNA discussed in the text. The position of transfected-cell-expressed TTP mRNA is indicated by an arrow. Film exposure was 7 h for the filters hybridized with mTNF-α and TTP cDNAs, as indicated; portions of the same blot probed with the TTP cDNA were also exposed to film for 18 h to show the expression of TTP mRNA in 293 cells transfected with low concentrations of CMV.hTTP.tag. The positions of the 18S rRNA are indicated. The blot hybridized with the mTNF-α cDNA was stripped and reprobed with a GAPDH cDNA probe; the filter was then exposed to film for 8 h and is shown at the bottom to demonstrate equivalent loading.

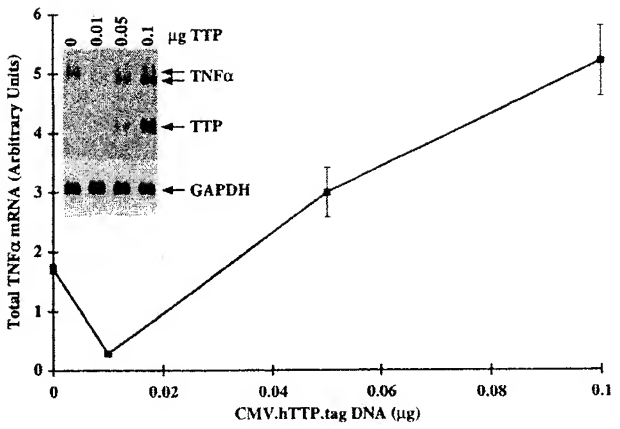


FIG. 3. Effect of low amounts of transfected CMV.hTTP.tag on the accumulation of TNF-α mRNA. 293 cells were cotransfected with CMV.mTNF-α and pXGH5 (1 μg each per plate) and either vector alone (5 μg/plate) or CMV .hTTP.tag (0.01, 0.05, and 0.1 μg/plate). Vector was also added to make the total amount of cotransfected plasmids 5 μg/plate. One day after replacement of the transfection medium, the medium was collected from each plate for the assay of released HGH. Total cellular RNA was then harvested. Each lane of the gel was loaded with 10 μg of total RNA. Electrophoresis and Northern blot hybridization were performed as described in Materials and Methods. The Northern blots were probed with either an mTNF-α cDNA probe or an mTTP cDNA probe and exposed to film. The blot that hybridized with the mTNF-α cDNA was stripped and reprobed with a GAPDH cDNA probe. The film showing the expressed mTNF-α mRNA was scanned with a laser scanning densitometer, and the results were normalized to HGH expression as well as to the PhosphorImager value for GAPDH mRNA. The graph shows the average results (± standard errors) from four such experiments; the inset shows the Northern blots from a representative experiment. The two species of TNF-α mRNA discussed in the text are indicated with two arrows; the positions of TTP mRNA and GAPDH mRNA are also indicated by arrows.

only one AUUUA motif, exhibited barely detectable TTP binding activity under these conditions (Fig. 1).

We therefore used CMV.mTNF-α in the cell expression studies described below, given the ability of TTP to bind to its mRNA ARE. The HEK 293 cells used in these studies normally do not express either TTP or TNF-α (Fig. 2, lane 0 or "Mock"), making these widely used cells a suitable intact cell system in which to study the interaction of TNF-α mRNAs with transfected-cell-expressed TTP. In addition, the expression of the truncated form of TNF-α mRNA in these cells made possible for the first time the detection of a processing (probably deadenylated; see below) intermediate; this intermediate was not detectable when the native, full-length TNF-α mRNA was expressed (data not shown).

Both TTP and TNF-α mRNAs were readily detected when the cells were transfected with either TTP or TNF-α expression plasmids (Fig. 2). The left side of Fig. 2 demonstrates the complex relationship that was found between the concentration of transfected CMV.hTTP.tag DNA and the resulting TNF-α mRNA accumulation in the absence of actinomycin D treatment. At low concentrations of transfected DNA (5 and 10 ng per plate [lanes 2 and 3 of Fig. 2, left]), TNF-α mRNA accumulation was ~20% of that of control, as determined by scanning densitometry of the Northern blot. This decrease in mRNA amount was accompanied by the appearance of a smaller species of mRNA, which first became apparent at 5 to 10 ng of DNA (Fig. 2, lanes 2 and 3, left) but was more obvious at 50 ng (Fig. 2, lane 4, left). As described below, we believe this lower band to be the deadenylated form of the TNF-α mRNA. Beginning at 50 ng of DNA (Fig. 2, lane 4, left) through all higher concentrations used (Fig. 2, lanes 5 to 8, left), essentially all TNF-α mRNA was in this smaller form. However, the total amount of TNF-α mRNA accumulation increased substantially at higher concentrations of DNA (see below) to reach a maximum of 214% of that of control at 500 ng (lane 6, left; $n = 4$ experiments). It remained high at 1 μg (lane 7, left; 200% of that of control; $n = 5$ experiments) before decreasing to 51% of that of control (lane 8, left; $n = 5$) at 5 μg. A similar but right-shifted dose-response relationship was present with the genomic TTP construct H6E.HGH3′, which uses the weaker native TTP promoter rather than the CMV promoter; in this case, 2 μg of DNA decreased total TNF-α mRNA accumulation to 16% of that of control ($n = 3$); higher concentrations (5 and 10 μg) resulted in continued expression of the smaller species in greater amounts.

The predominance of the smaller band and the almost complete absence of the larger band could be seen more readily after actinomycin D exposure (right side of Fig. 2), presumably because the larger band represented recently synthesized TNF-α mRNA that was more likely to be full length. In this case, 5 and 10 ng of CMV.hTTP.tag DNA resulted in less than 10% of control TNF-α mRNA expression (Fig. 2, lanes 2 and 3, right).

Because of the peculiar nature of this dose response, we performed four identical experiments with low concentrations of CMV.hTTP.tag, in which all samples were corrected for transfection efficiency with HGH expression and were corrected for loading by Northern blot analysis of GAPDH mRNA and PhosphorImager analysis (Fig. 3). An example of

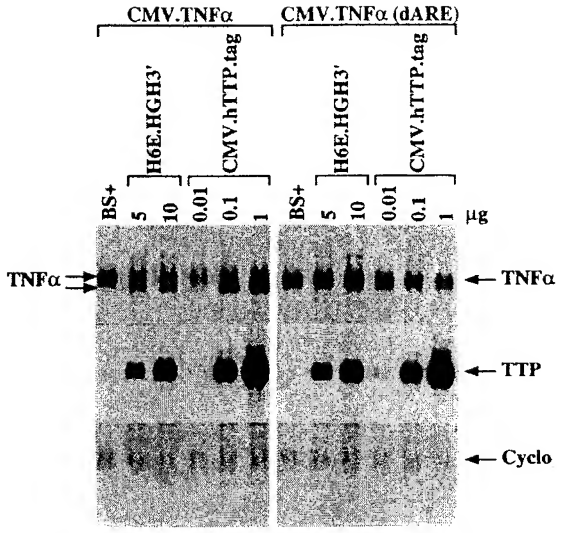


FIG. 4. Effect of TTP expression on the expression of TNF-α constructs containing or lacking the ARE. CMV.mTNF-α (1 μg/plate) or CMV.mTNF-α (dARE) (1 μg/plate) was cotransfected into 293 cells with either vector alone (BS+, 5 μg/plate), H6E.HGH3′ (5 or 10 μg/plate), or CMV.hTTP.tag (0.01, 0.1, or 1 μg/plate). Vector was also added to make the total amount of cotransfected plasmids 5 μg/plate. Preparation of total cellular RNA, electrophoresis, and Northern blot analysis were performed as described in Materials and Methods. Each lane was loaded with 10 μg of total RNA. The Northern blots were probed with an mTNF-α cDNA, together with probes for cyclophilin (Cyclo) or mTTP as indicated. On the left, the arrows indicate the two species of mTNF-α mRNA formed in the presence of TTP; on the right, the arrow indicates the single band of mTNF-α mRNA expressed from the plasmid lacking the ARE. TTP mRNA expressed from the cotransfected plasmid and the endogenous cyclophilin mRNA are also indicated by arrows.

one such experiment is shown in the inset in Fig. 3, and the mean values ± standard errors from the four experiments are indicated in Fig. 3. Compared to the vector-alone control, there was a decrease in total hybridizing TNF-α mRNA by 83% (to 17% of that of control) at 0.01 μg of CMV.hTTP.tag. This mean value increased to 173% of that of control at 0.05 μg and to 300% of that of control at 0.1 μg of DNA. As shown in the inset and in Fig. 2, most of the hybridizing TNF-α mRNA seen at the higher concentration of transfected CMV.hTTP.tag was in the smaller form.

To determine whether transcription of CMV.TNF-α was affected by the TTP expression plasmids, various amounts of either H6E.HGH3′ or CMV.hTTP.tag were cotransfected into 293 cells with CMV.mTNF-α or CMV.mTNF-α (dARE). In the latter construct, which was otherwise identical to CMV.mTNF-α, 24 bp of the ARE was deleted (bp 1302 to 1325 of mTNF-α cDNA [Fig. 1]), resulting in a disrupted ARE that was incapable of binding TTP (see below). In this case, despite equivalent coexpression of TTP, the TNF-α mRNA expressed from the CMV.mTNF-α construct containing the normal ARE was shortened in the normal way by the coexpressed TTP (Fig. 4, left), while expression of the mutated CMV.mTNF-α construct was unaffected either in apparent size or in total accumulation by any concentration of cotransfected H6E.HGH3′ and was minimally affected by CMV.hTTP.tag (Fig. 4, right). Quantitation of these result by PhosphorImager analysis and normalization for loading by cyclophilin mRNA showed that H6E.HGH3′ at 5 and 10 μg resulted in TNF-α (dARE) expression that was 105 and 98%, respectively, of that of the vector-alone cotransfected control, whereas CMV.hTTP.tag at 0.01, 0.1, and 1 μg resulted in TNF-α (dARE) expression that was 110, 97, and 73% of that of control, respectively. These experiments indicate that the effect of TTP in decreasing TNF-α mRNA accumulation at low concentrations of CMV.hTTP.tag (i.e., 5 and 10 ng) was unlikely to be due to nonspecific squelching of transcription (7, 34), although this may have contributed to the modest decrease in TNF-α mRNA expression seen with larger (5 μg) amounts of CMV.hTTP.tag.

Evidence that TTP promoted deadenylation of TNF-α mRNA. The effect of TTP expression in causing shortening of the TNF-α mRNA suggested that TTP was promoting deadenylation of the TNF-α mRNA poly(A) tail. To evaluate this possibility, oligo$(dT)_{12-18}$ (P1) was added to total cellular RNA, and RNase H was used to remove the poly(A) tail (31). When this technique was used on RNA samples from cells cotransfected with CMV.mTNF-α and either vector alone or TTP expression constructs (H6E.HGH3′ in Fig. 5A and CMV.hTTP.tag in Fig. 5B and C), only the smaller of the two TNF-α mRNA species remained (Fig. 5A and B; P1, lanes 1 and 2). The smaller of the two mRNA species seen in the cells transfected with TTP constructs did not further decrease in size with the RNase treatment; this fact, and its identity in size to the deadenylated TNF-α mRNA from the control cells, indicated that the smaller form of the TNF-α mRNA was deadenylated mRNA.

We also performed an RNase H experiment that used an oligonucleotide complementary to bp 506 to 528 of TNF-α mRNA (GenBank accession no. X02611; P2). The predicted sizes of the mRNA fragments from the resulting mRNA cleavage were ~400 bp (5′ portion) and ~810 bp (3′ portion) (Fig. 5A, P2, lanes 1 and 2; Fig. 5B and C, P2, lanes 3 and 4). When RNA from 293 cells expressing both TNF-α and TTP was analyzed after cleavage, most of the 3′ TNF-α mRNA fragment was in the form of the deadenylated smaller species, compared to RNA harvested from cells expressing TNF-α and vector alone (Fig. 5). When both oligonucleotides were added together (Fig. 5C, P1 + P2), the 3′ fragment of the TNF-α mRNA was of identical size in samples from control (lane 3) and TTP (lane 4)-expressing cells. The size of the ~400-bp 5′ fragment was unaffected by TTP expression (Fig. 5). These data confirmed that TTP promoted deadenylation of the TNF-α mRNA.

Evidence for a precursor-product relationship between the upper and lower forms of TNF-α mRNA. In order to demonstrate that the larger, presumably polyadenylated form of TNF-α mRNA could be converted to the smaller, deadenylated form by the presence of TTP, we analyzed the patterns of TNF-α mRNA expression in cells cotransfected with small amounts of TTP expression constructs, before and after 4 h of exposure to actinomycin D (10 μg/ml). As shown in the cells transfected with vector alone (BS+, 10 μg; BS+, 5 μg), there was no conversion of the larger form of TNF-α mRNA to a stable, smaller form in the absence of TTP, although the total amount of full-length mRNA decreased modestly after 4 h of actinomycin D exposure (Fig. 6A). However, in the presence of TTP (10 μg of H6E.HGH3′ or 0.05 to 0.5 μg of CMV .hTTP.tag), actinomycin D exposure clearly led to the disappearance of the larger band, so that only the smaller band remained (Fig. 6A). Two additional experiments also examined intermediate time points. Figure 6B shows the time course of disappearance of the upper band in the presence of TTP (10 μg of H6E.HGH3′ transfected) after 0, 2, and 3 h of actinomycin D treatment. Figure 6C shows a longer time course after expression of CMV.hTTP.tag (0.1 μg) with 0, 4, and 8 h of actinomycin D treatment. In both cases, the expression of TTP resulted in both forms of TNF-α mRNA; the

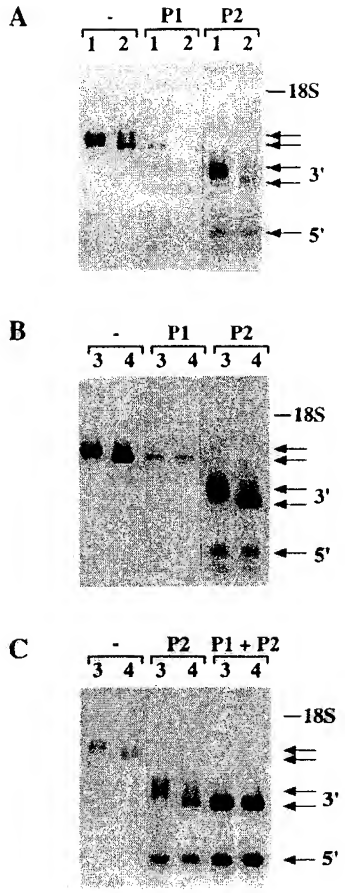


FIG. 5. Evidence that the smaller species of TNF-α mRNA formed in the presence of TTP is a deadenylated intermediate. 293 cells were cotransfected with CMV.mTNF-α (1 μg/plate) and either vector alone or TTP expression constructs as follows: lanes 1, vector alone (10 μg/plate); lanes 2, H6E.HGH3′ (10 μg/plate); lanes 3, vector alone (5 μg/plate); lanes 4, CMV.hTTP.tag (5 μg/plate). As indicated, the RNA samples were treated with 0.8 U of RNase H or not treated (−) as described in Materials and Methods. In panels A and B, 5 μg of RNA was loaded into each lane; in panel C, 3 μg of RNA was loaded into each lane when no RNase H (−) was used. P1, oligonucleotide poly(dT)$_{12-18}$ (0.5 μg) was added to 10 μg of 293 cell RNA. P2, an oligonucleotide (0.6 μg) complementary to bases 506 to 528 of the TNF-α mRNA was added to 10 μg of 293 cell RNA. P1 + P2, both oligonucleotides were added to 15 μg of 293 cell RNA (C). The Northern blots were probed with an mTNF-α cDNA. The position of the 18S rRNA is indicated. The two pairs of arrows in each panel indicate TNF-α mRNA species that contained (top arrow of each pair) or did not contain (bottom arrow of each pair) their poly(A) tails. The top pair of arrows in each panel points to full-length and deadenylated TNF-α mRNA; the bottom pair of arrows in each panel (3′) points to the 810-bp 3′ fragment of TNF-α mRNA and its deadenylated form in lanes P2-2 and P2-4. The single arrow at the bottom (5′) indicates the ~400-bp 5′ fragment of TNF-α mRNA, which is the same size in control and in TTP-expressing cells.

upper form then gradually disappeared after actinomycin D treatment.

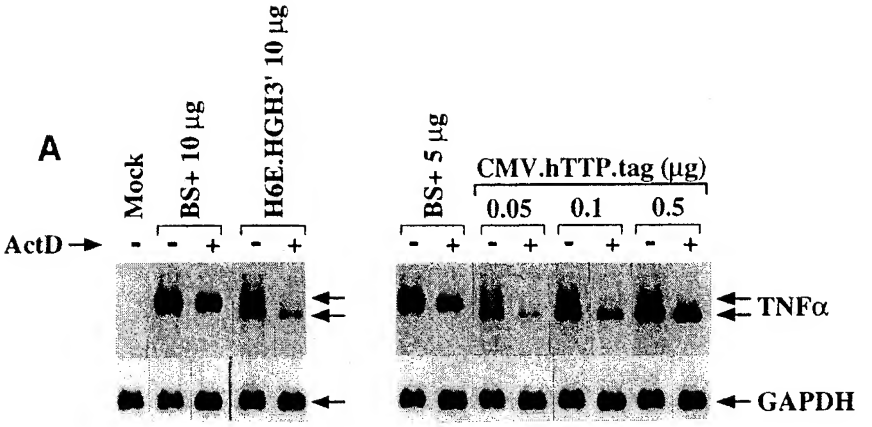
Evidence that the ARE-binding protein in 293 and macrophage extracts is TTP. We next examined TNF-α ARE-binding activity in cytosolic extracts from bone marrow-derived macrophages from wild-type and TTP$^{-/-}$ mice that had been stimulated with LPS. After the cell extracts were UV cross-linked to the TNF-α ARE probe and treated with RNases, an RNase-resistant RNA-protein complex was immunoprecipitated by an anti-TTP antibody but not by preimmune serum (Fig. 7). The macrophage TTP that was immunoprecipitated from the LPS-treated TTP$^{+/+}$ cells, but not from untreated TTP$^{+/+}$ cells or from the treated or untreated TTP$^{-/-}$ cells, appeared as a smear with an average size of ~50 kDa, compared to the apparent 40 to 44 kDa of mTTP expressed from CMV.mTTP in 293 cells (Fig. 7). In our earlier studies, TTP migrated as a smear or multiple bands of ~35 to ~55 kDa (7, 44, 45). The difference in apparent molecular masses seen in the present experiment may have been due to differences in posttranslational modification of the TTP protein, since, for example, its apparent molecular mass is known to increase after mitogen-stimulated phosphorylation (44). Despite these differences in apparent M_r, the identity of the immunoprecipitated protein as TTP cross-linked to ^{32}P-labeled TNF-α ARE was confirmed by the facts that the complex was precipitated from 293 cells that were transfected with TTP-expressing plasmids but not from cells transfected with vector alone, it was precipitated from 293 cells by three different antibodies including an antibody to the epitope tag (7), and it was specifically immunoprecipitated from LPS-stimulated wild-type macrophages but not from unstimulated wild-type cells or from stimulated or unstimulated TTP-deficient cells.

These results indicate that the endogenous TTP formed after LPS treatment of normal macrophages can also bind to the TNF-α ARE and support the previously documented connection between the expression of TTP and the more rapid decay of TNF-α mRNA in macrophages (7).

Involvement of the TTP zinc fingers in the binding of TTP to the ARE of TNF-α mRNA. We next evaluated the possible involvement of each of the two CCCH zinc fingers in the ARE-binding activity of TTP, by using the TNF-α probe 1197–1350 (Fig. 1). When cell extracts prepared from 293 cells that had been transfected with vector alone were used in UV cross-linking experiments, a major radioactive band of ~80,000 in M_r and several minor species were noted (Fig. 8A, lane 1). When cell extracts prepared from 293 cells that had been transfected with hTTP expression constructs were used in UV cross-linking experiments, the extracts from cells transfected with either the wild-type CMV.hTTP.tag (lane 3) or the S228A mutant (a point mutation at a MAP kinase phosphorylation site in the protein [44]) (lane 6) formed readily detectable RNase-resistant RNA-protein complexes of ~43,000 in M_r with the ^{32}P-labeled TNF-α RNA probe, while simultaneously decreasing binding of the ARE probe to the endogenous cellular ~80,000-M_r protein. However, extracts from cells transfected with 10 μg of H6E.HGH3′ (hTTP driven by its native promoter and intron) (lane 2), with the C124R mutation in the CMV.hTTP.tag construct (the third C in the first zinc finger mutated to an R; lane 4), or the C147R mutation in CMV .hTTP.tag (the first C in the second zinc finger mutated to an R; lane 5) exhibited no detectable ARE-binding activity. This indicates that single cysteine-to-arginine mutations in each of the TTP zinc fingers completely prevented TTP binding to the TNF-α ARE.

When the same UV cross-linked, RNase-treated extracts from cells transfected with CMV.hTTP.tag (lanes 3) or H6E.HGH3′ (lanes 2) were immunoprecipitated with a polyclonal antibody to hTTP (DU88), or with a polyclonal antibody to mTTP (2640), an RNA-protein complex of 40,000 to 50,000 in M_r was precipitated (Fig. 8B). This indicates that the failure to see binding of TTP to the TNF-α ARE probe in crude extracts from H6E.HGH3′-transfected cells (Fig. 8A, lane 2) was simply due to much lower expression of the construct relative to the CMV construct. Neither antibody immunoprecipitated complexes from cells transfected with vector alone (lanes 1).

When the same UV cross-linked, RNase-treated extracts



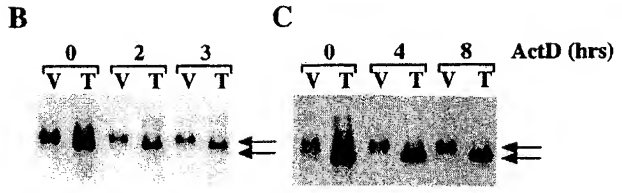


FIG. 6. Effect of TTP on the formation of the two species of TNF-α mRNA in the presence and absence of actinomycin D. CMV.mTNF-α (1 μg/plate) was cotransfected into 293 cells with either TTP expression constructs, as indicated, or vector alone (BS+). After addition of buffer alone (−) or actinomycin D (ActD) to a final concentration of 10 μg/ml (+), total cellular RNA was harvested. Each lane was loaded with 10 μg of total RNA. Electrophoresis and Northern blot hybridization were performed as described in Materials and Methods. (A) (Left) TNF-α and GAPDH mRNA from mock-transfected 293 cells or from cells cotransfected with CMV.mTNF-α and vector alone (10 μg/plate) or CMV.mTNF-α with TTP expression construct H6E.HGH3′ (10 μg/plate). (Right) TNF-α and GAPDH mRNA from 293 cells cotransfected with CMV.mTNF-α and vector alone (5 μg/plate) or CMV.mTNF-α with CMV.hTTP.tag (0.05, 0.1, and 0.5 μg/plate). Vector was also added to make the total amount of cotransfected plasmids 5 μg/plate. Actinomycin D (+) was added for 4 h as indicated. The two arrows labeled TNF-α indicate the two species of TNF-α mRNA formed in the presence of TTP. (B) H6E.HGH3′ (T) (10 μg/plate) or an equivalent amount of vector (V) was used in the cotransfection, and actinomycin D (10 μg/ml) was added for 0, 2, and 3 h as indicated. (C) CMV.hTTP.tag (T) (0.1 μg/plate) or an equivalent amount of vector (V) was used, and actinomycin D (10 μg/ml) was added for 0, 4, and 8 h as indicated. The two arrows indicate the two forms of TNF-α mRNA in panels B and C.

were immunoprecipitated with a polyclonal antibody to the epitope tag on TTP, the same RNA-protein complexes were precipitated from cells transfected with either the wild-type CMV.hTTP.tag (lane 3) or the S228A mutant (lane 6), but only barely detectable complexes were seen in extracts from the cells transfected with either of the two zinc finger mutants in the CMV.hTTP.tag construct (lanes 4 and 5). Note in both Fig. 8B and C the appearance of an immunoprecipitated complex of ~100,000 in M_r; this is clearly recognized by both antibodies to TTP and to the epitope tag and most likely represents either TTP dimers or TTP complexed to a second protein of similar size as well as to the TNF-α ARE probe.

To determine whether the mutant constructs used in these experiments expressed amounts of TTP protein that were equivalent to those expressed by the wild-type constructs, extracts prepared from 293 cells transfected with equivalent amounts of vector alone (lane 1) or either wild-type (lane 3) or mutant plasmids (lanes 4 to 6, 5 μg of each) were subjected to Western blotting (Fig. 8D). Comparable amounts of fusion proteins were expressed from all four constructs, as recognized by the antibody to the epitope tag HA.11. An immunoreactive protein of ~100,000 in M_r was also seen by this technique, indicating that the integrity of the two zinc fingers in TTP is not required for the formation of these higher-M_r complexes, whether they are TTP dimers or TTP bound to another protein.

To further demonstrate that the binding of TTP to the TNF-α ARE was specific, we made a mutant probe of pTNF-α 1309–1332 in which five of the flanking A's in the AUUUA motif of the ARE sequence were mutated to G's (Fig. 1). When this radiolabeled mutant probe was UV cross-linked to the extract from CMV.hTTP.tag-transfected 293 cells, there was no detectable formation of the TTP complex, while the amount of the ~80,000-M_r complex was decreased but not eliminated (data not shown). In contrast, the wild-type probe 1309–1332 could be readily cross-linked to TTP (not shown).

Electrophoretic mobility shift assays. The specificity of TTP binding to the TNF-α ARE was also analyzed by electrophoretic mobility shift assays with TNF-α 3′ UTR probes. Incubation of probe 1197–1350 (containing the seven AUUUA motifs and some sequence 5′ to them [Fig. 1]) with a cytosolic extract prepared from 293 cells transfected with vector alone resulted in three major RNA-protein complexes, labeled I, II, and III (Fig. 9A, lane 1). When extracts from cells transfected with hTTP expression constructs were used, there were changes in the mobility of RNA-protein complexes I and II, while complex III disappeared (Fig. 9A, lanes 3, 2, and 6). In a separate experiment, the extract from control cells was incubated with probe 1197–1350, and RNA-protein complexes were separated in a mobility shift assay. After the gel was exposed to UV light, complexes I, II, and III were eluted and analyzed by SDS-PAGE. Complexes I and II corresponded to an ~80-kDa protein, and complex III corresponded to a ~55-kDa protein (data not shown). In the mobility shift assays, the TTP-probe complex migrated in approximately the same positions as did complexes I and II (as noted above for the UV cross-linking assays, the binding of TTP to the TNF-α mRNA ARE simultaneously decreased the binding of the ARE probe to the endogenous cellular ~80,000-M_r protein). The difference in appearance between lanes 3 and 2 was probably due to the amounts of TTP protein expressed by the different expression vectors. In lane 3, the extract was from 293 cells transfected with 10 μg of H6E.GHG3′, a construct that uses the weaker native promoter and produces the native TTP protein;

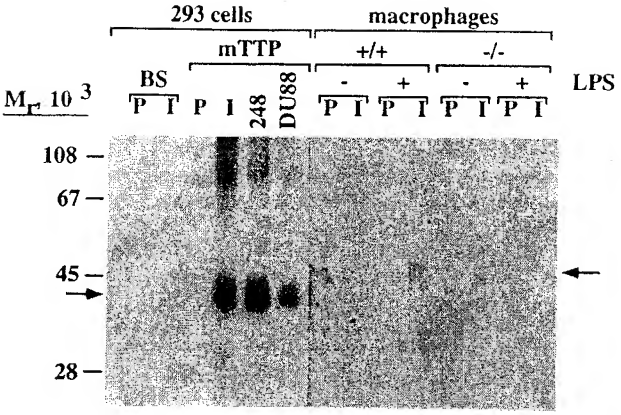


FIG. 7. UV cross-linking and immunoprecipitation of TTP-RNA complexes from 293 cells or $TTP^{+/+}$ or $TTP^{-/-}$ macrophages. Cytosolic extracts from 293 cells transfected either with CMV.mTTP (5 μg) or with vector (BS) alone (5 μg) were prepared as described in Materials and Methods. Cytosolic extracts from $TTP^{+/+}$ or $TTP^{-/-}$ macrophages untreated (−) or treated with (+) 1 μg of LPS per ml for 4 h were prepared as described elsewhere (7). Incubation of extracts (each sample contained 20 μg of protein from 293 cells or 40 μg of macrophage protein) with the ^{32}P-labeled TNF-α probe (1281–1350), UV cross-linking, and RNase digestion were performed as described in Materials and Methods. The samples were then precleared with preimmune serum and divided into two portions, which were then incubated with preimmune serum (P), a polyclonal antibody to an mTTP–glutathione *S*-transferase fusion protein (I), a polyclonal antibody raised against an amino-terminal peptide of mTTP (248), or a polyclonal antibody to an hTTP–glutathione *S*-transferase fusion protein (DU88). The immunoprecipitated complexes were resolved by SDS-PAGE (10% polyacrylamide gel) and autoradiography. Film exposure was 8 h for the 293 cell gel and 6 days for the macrophage gel. The positions of radiolabeled transfected-cell-expressed TTP (293 cells) and endogenous macrophage TTP are indicated by the arrows. The positions of protein molecular weight standards are indicated.

in lanes 2 and 6, epitope-tagged TTP was expressed with the stronger CMV promoter. The same changes in protein-probe complex formation were seen when probes 1110–1325 (containing four AUUUA motifs [Fig. 1]), 1281–1350 (containing seven AUUUA motifs), and 1309–1332 (containing only four clustered UUAUUUAUU nanomers) were used in the same assay (data not shown). In order to demonstrate that the binding of complexes I and II, and TTP, to the TNF-α ARE probes was specific, we also used a 54-nucleotide region from the c-*fos* 3′ UTR that has a 62% AU content without any AUUUA motifs (53) in the mobility shift assay. This 54-nucleotide probe did not form complexes I and II with cytosolic extracts prepared from 293 cells transfected with vector alone, nor did it form a binding complex with extracts from TTP-expressing cells (data not shown).

When one of the cysteine residues in either the first or the second zinc finger was mutated in construct CMV.hTTP.tag, extracts prepared from 293 cells transfected with these mutants no longer changed the mobility pattern of complexes formed when probe 1197–1350 was used (Fig. 9A, lanes 4 and 5). Similar results were obtained when probe 1110–1325 or 1281–1350 was used (data not shown).

To demonstrate that the mobility changes in complexes I and II were due to the binding of TTP to the TNF-α RNA probe, an antibody to the epitope tag of the TTP fusion protein was added to the mobility shift assay (Fig. 9B). Although the antibody did not change the migration pattern of the RNA-protein complexes in extracts from control cells (lanes 1) or from cells transfected with the two TTP zinc finger mutants (lanes 4 and 5), it retarded the migration of complexes formed in extracts from cells expressing either wild-type TTP (lanes 2) or its S228A mutant (lanes 6). This supershift of the binding complex provided additional confirmation that the protein that bound to the RNA was TTP.

Importantly, the absence of TNF-α ARE-binding activity of the two TTP zinc finger mutants corresponded to their lack of effect on the conversion of TNF-α mRNA to the smaller species in 293 cells (Fig. 10). Normal amounts of the larger species of TNF-α mRNA were present when CMV.mTNF-α was cotransfected with either of the two TTP zinc finger mutant constructs, driven either by the CMV or by the native hTTP promoter (lanes 4, 5, 10, and 11). The MAP kinase phosphorylation site mutant S228A, which retained its ability to bind to the TNF-α ARE, also behaved like native TTP in promoting the shift to the smaller species of TNF-α mRNA (lane 3) in intact 293 cells.

These experiments demonstrated the importance of the integrity of each of the zinc fingers in the binding of TTP to the TNF-α ARE, as well as in the apparent deadenylation of the TNF-α mRNA. These assays also indicated the importance of multiple cysteines in the zinc fingers, since mutating either the third C in the first finger or the first C in the second finger abolished TTP's RNA binding and cleavage-promoting activity.

TTP is largely nonnuclear in these experiments. We previously demonstrated by differential centrifugation techniques that TTP was almost exclusively cytosolic in normal mouse macrophages (7) and in the macrophage cell line RAW 264.7 (45), although it had previously been localized to the nucleus of both quiescent (11, 45) and serum-stimulated (11) fibroblasts. For the present study, we constructed plasmids that expressed human TTP as a fusion protein with a modified GFP, which normally is distributed throughout the cytoplasm and nucleus; this modified GFP localizes within the cell based on the peptides fused to it (12, 39). When 293 cells were transfected with EGFP-N1 (GFP alone driven by the CMV promoter), fluorescence was present in both the nucleus and the cytoplasm (Fig. 11A). However, when the TTP-GFP fusion construct was transfected into 293 cells, the fluorescence was somewhat heterogeneous and appeared to be largely nonnuclear. This was true in cells transfected with both CMV .hTTP.EGFP (Fig. 11C to F) and the H6E.EGFP construct (Fig. 11G and H) in which the hTTP-GFP fusion protein expression was driven by the native hTTP promoter and intron. Both the promoter and the single intron of TTP play important roles in its expression (23, 24). Similar predominantly cytosolic distribution was seen in HeLa cells transfected with the same constructs (data not shown).

To determine whether the hTTP-GFP fusion protein expressed in 293 cells was biologically active in these cells, we tested its ability to bind to the TNF-α ARE probe in the cell-free assay mixtures and to promote the size shift of TNF-α mRNA in the intact cells (Fig. 12). Both activities were exhibited by the hTTP-GFP fusion protein (Fig. 12, lanes 2 and 3). We also demonstrated that a single C-to-R mutation in either the first or the second zinc finger of hTTP markedly inhibited the ability of this hTTP-GFP fusion protein to cause the size shift in TNF-α mRNA in 293 cells or to bind to the TNF-α ARE in cell extracts (Fig. 12, lanes 4 and 5). These mutations did not appear to affect the pattern of distribution of the protein in the cells (data not shown).

DISCUSSION

The experiments reported here identify a probable function for TTP, the prototype of a subclass of vertebrate CCCH proteins characterized by two closely spaced zinc fingers with a characteristic lead-in sequence for each finger.

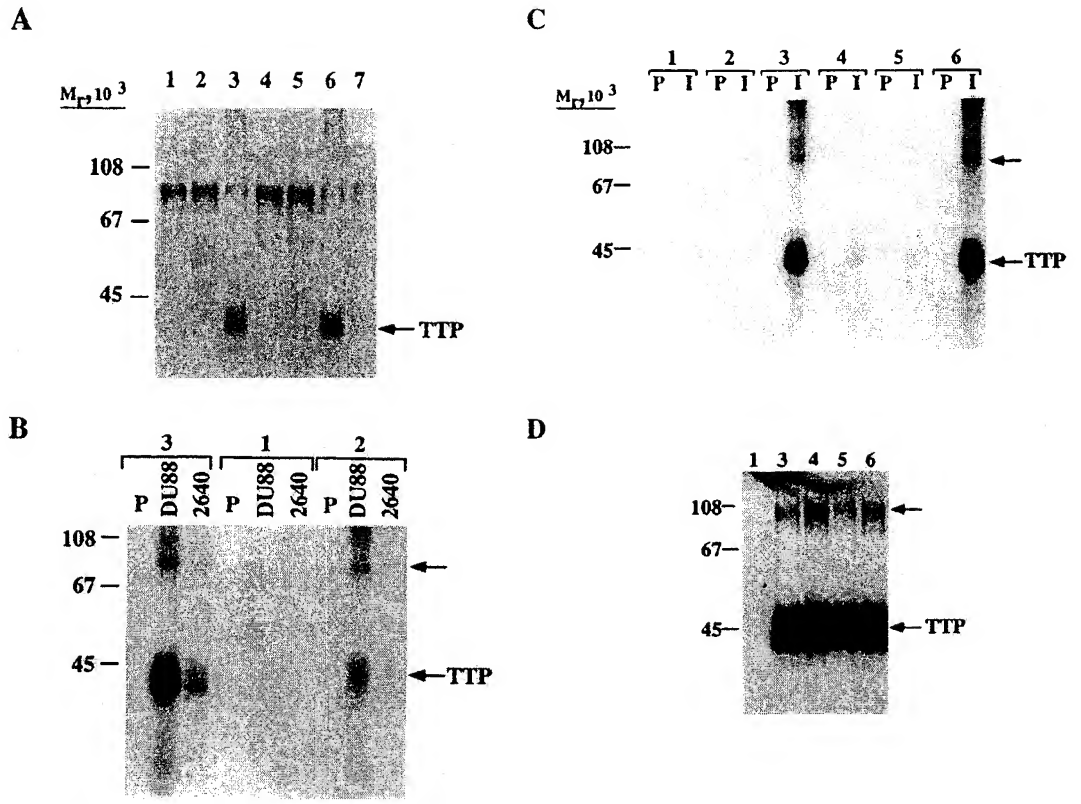


FIG. 8. UV cross-linking assays of TTP–mTNF-α–ARE complexes. Cytosolic extracts of 293 cells transfected with either vector alone or constructs expressing hTTP were prepared as described in Materials and Methods. (A) Lanes 1, extracts from 293 cells transfected with 5 μg of vector plasmid; lanes 2, extracts from 293 cells transfected with 10 μg of plasmid H6E.HGH3′; lanes 3 to 6, extracts from 293 cells transfected with 5 μg of wild-type CMV.hTTP.tag (lane 3), zinc finger mutant C124R (lane 4), zinc finger mutant C147R (lane 5), or phosphorylation site mutant S228A (lane 6); lane 7, TNF-α probe (1197–1350) alone (5,000 cpm). UV cross-linking and RNase digestion were performed as described in Materials and Methods. The RNA-protein complexes were resolved by SDS-PAGE (10% polyacrylamide gel) followed by autoradiography. The bands at ~42,000 M_r in lanes 3 and 6 (arrow) represent radiolabeled TTP. Note the endogenous cellular protein of ~80,000 in M_r that is cross-linked to the TNF-α ARE in the absence of expressed TTP; this cross-linking was markedly decreased in the presence of TTP (lanes 3 and 6). (B) UV cross-linked and RNase-digested samples as described for panel A (lanes 1 to 3) were precleared with preimmune serum and divided into three portions that were incubated with preimmune serum (P), a polyclonal antibody to an hTTP–glutathione *S*-transferase fusion protein (DU88), or a polyclonal antibody to an mTTP–glutathione *S*-transferase fusion protein (2640). The immunoprecipitated complexes were resolved by SDS-PAGE (10% polyacrylamide gel) and autoradiography. The higher-molecular-weight immunoprecipitated complex is indicated by the upper arrow, and the position of TTP is indicated by the lower one. (C) UV cross-linked and RNase-digested samples as described for panel A (lanes 1 to 6) were precleared with preimmune serum and divided into two portions that were incubated with preimmune serum (P) or a polyclonal anti-epitope tag antibody (I). The immunoprecipitated complexes were resolved by SDS-PAGE (10% polyacrylamide gel) and autoradiography. A higher-molecular-weight immunoprecipitated complex is indicated by the upper arrow, and the position of TTP is indicated by the lower one. (D) Five micrograms of protein extract prepared from 293 cells as described above (lanes 1 and 3 to 6) was analyzed by Western blotting with a polyclonal antibody to the epitope tag of the fusion protein hTTG.tag. The positions of molecular weight standards (in thousands) are indicated to the left of each gel. The position of TTP is indicated by the lower arrow; the position of the ~100,000-M_r complex that contains TTP is indicated by the upper arrow.

This protein exhibited several activities in our assays. In a cell-free system, TTP bound directly to the ARE from the TNF-α mRNA. This binding was dependent upon the integrity of both zinc fingers, in that mutation of a single cysteine to arginine in either zinc finger almost totally abrogated binding to the ARE, as determined by both UV light cross-linking and gel mobility shift experiments. In intact cells, the protein caused decreases in the apparent size of TNF-α mRNA in cotransfection experiments; again, this effect was not seen with cotransfection of either single amino acid zinc finger mutant. The cotransfection experiments coupled with RNase H analysis pointed to an action of TTP in stimulating removal of the poly(A) tail of the mRNA, leading to the formation of a deadenylated intermediate. Under appropriate conditions, i.e., at low concentrations of expressed TTP, this species was also degraded to yield a net decrease in total hybridizable TNF-α mRNA accumulation.

We postulate, therefore, that TTP can participate in the series of steps comprising the initial deadenylation followed by the ultimate degradation of at least some of those mRNAs containing so-called type II AREs (8, 37), exemplified by TNF-α, GM-CSF, and IL-3 (53). It seems likely that an early or possibly the first step in this interaction is the direct, zinc finger-mediated binding of TTP to the ARE, followed by a series of unknown steps that leads ultimately to removal of the poly(A) tail and subsequent (or simultaneous) mRNA degradation. That these events are likely to be physiologically significant is indicated by the results of our earlier studies with the TTP knockout mice and macrophages derived from them, in which the mice developed a TNF-α excess syndrome associated with increased macrophage production of TNF-α, due at least in part to increased stability of the TNF-α mRNA in the cells (6, 7, 46).

Many of our conclusions concerning the TTP-stimulated removal of the poly(A) tail rely on data from an unnatural construct, i.e., CMV.mTNF-α, in which the TNF-α ARE, normally 277 bp 5′ of the poly(A) tail, is immediately 5′ of a synthetic tail of 33 A residues directly encoded by the trans-

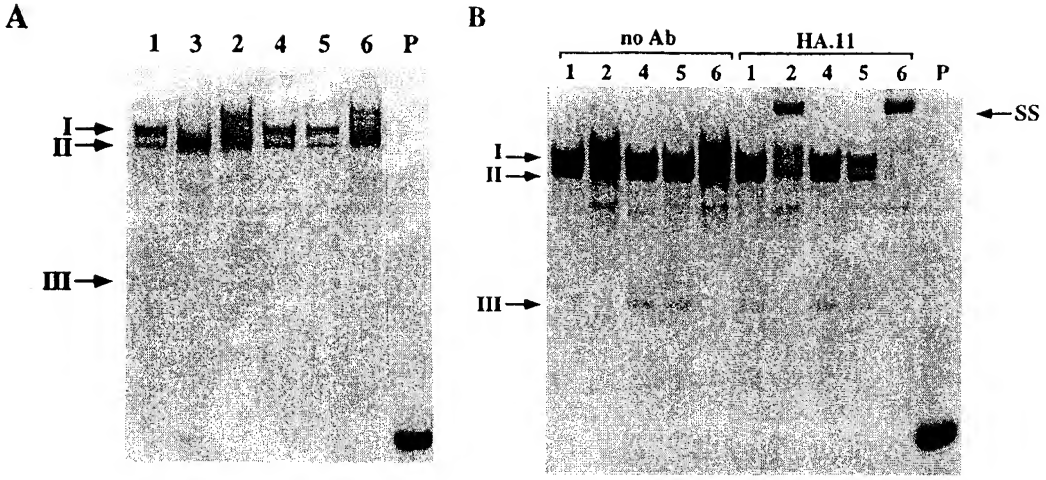


FIG. 9. Electrophoretic mobility shift assays of TTP–TNF-α–ARE complexes. Cytosolic extracts of 293 cells transfected with either vector alone or constructs expressing hTTP were prepared as described in Materials and Methods. Ten micrograms of protein extract was used in the incubation with 10^5 cpm of a TNF-α ARE probe, and RNA mobility shift assays were performed as described in Materials and Methods. Lanes 1, extracts from 293 cells transfected with 5 μg of vector plasmid; lanes 3, extracts from 293 cells transfected with 10 μg of plasmid H6E.HGH3′; lanes 2 and 4 to 6, extracts from 293 cells transfected with 5 μg of wild-type CMV.hTTP.tag (lane 2), zinc finger mutant C124R (lane 4), zinc finger mutant C147R (lane 5), or phosphorylation site mutant S228A (lane 6). (A) RNA-protein complex migration patterns were compared in the presence of wild-type TTP (lanes 2 and 3) and its zinc finger mutants (lanes 4 and 5) or its phosphorylation site mutant (lane 6). P, mTNF-α probe alone (1197–1350) after digestion with RNase T_1. (B) RNA-protein complex migration patterns were compared as described for panel A, in the absence (no Ab) or presence (HA.11) of a polyclonal anti-epitope tag antibody. The supershifted RNA-protein complexes (SS) are indicated by the arrow. RNA-protein complexes I, II, and III are indicated in both panels. P, mTNF-α probe alone (1197–1350) after digestion with RNase T_1.

fected cDNA. This construct allowed us to compare the results of cotransfection with TTP to those from the RNase H experiments and permitted the conclusion that one of the results of TTP coexpression was the removal of this synthetic tail. This incremental decrease in size of the synthetic TNF-α message was not seen with the two constructs containing single amino acid mutations within the zinc fingers. In contrast, transfection studies in which these proteins were coexpressed with mRNAs consisting of β-globin-coding sequences and 3′ UTR, within which the AREs from TNF-α, GM-CSF, and IL-3 were inserted, as well as studies using the native TNF-α mRNA, did not appear to result in the formation of stable deadenylated forms of the mRNA. Instead, coexpression of TTP resulted in a decrease in the steady-state levels of the mRNAs, without the accumulation of stable truncated intermediates (7). We suggest that, under normal circumstances, TTP binding to the ARE of these mRNAs results in the same series of reactions, i.e., deadenylation followed by continued mRNA degradation, without the formation of easily detectable stable intermediates; this has been demonstrated in macrophages from $TTP^{+/+}$ and $TTP^{-/-}$ mice, in which stable mRNA intermediates were not detectable in cells stimulated with LPS and then exposed to actinomycin D (7).

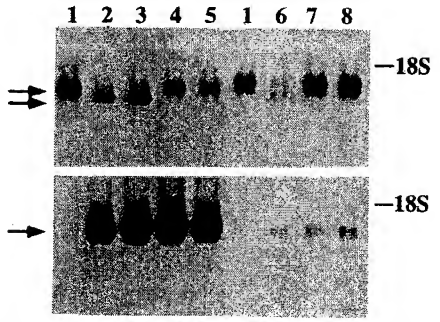


FIG. 10. TTP zinc finger mutants are ineffective at promoting the size shift of TNF-α mRNA. CMV.mTNF-α (1 μg/plate) was cotransfected into 293 cells with 5 μg of vector alone per plate (lanes 1) or 5 μg of wild-type CMV.hTTP.tag (lane 2), phosphorylation site mutant S228A (lane 3), zinc finger mutant C124R (lane 4), or zinc finger mutant C147R (lane 5). For lanes 6 to 8, 293 cells were transfected with 10 μg of wild-type plasmid H6E.HGH3′ (lane 6) or its zinc finger mutants (C124R, lane 7; C147R, lane 8). Preparation of total cellular RNA, electrophoresis, and Northern blot analysis were performed as described in Materials and Methods. Each lane was loaded with 10 μg of total RNA. The Northern blots were probed with a ^{32}P-labeled mTNF-α cDNA (upper panel) or mTTP cDNA (lower panel). In the upper panel, the arrows indicate the two species of mTNF-α mRNA. In the lower panel, the arrow indicates the position of transfected-cell-expressed TTP mRNA. The position of the 18S rRNA is indicated.

Another problematic aspect of our data concerns the triphasic dose-response curve of TNF-α mRNA expression as a function of expressed TTP. At low levels of transfected CMV.hTTP.tag (10 ng of DNA/plate of cells), coexpression of TTP caused a decrease of 83% in total hybridizing TNF-α mRNA. However, at higher CMV.hTTP.tag DNA concentrations, although the hybridizing species of TNF-α mRNA was the smaller, deadenylated form, there was a clear-cut increase in the amount of hybridizing TNF-α mRNA, reaching about 300% of control at 100 ng of CMV.hTTP.tag DNA. This net increase in total hybridizing TNF-α mRNA at higher transfected DNA concentrations was seen in more than 10 independent experiments. We propose that the physiologically relevant effect of TTP is to cause the deadenylation and subsequent degradation of the TNF-α mRNA, as seen with low amounts of expressed TTP. However, at higher TTP concentrations, although deadenylation still occurred, TTP clearly exerted a protective effect on the remaining deadenylated mRNA body. This could be due to continued TTP binding to the ARE of the deadenylated mRNA body, which could protect the body from further degradation. Alternatively, the excess TTP in the cells could be binding to and inhibiting the activity of other proteins involved in the degradation of the mRNA body, such as exonucleases or endonucleases. We favor the second explanation because of an apparent dominant-neg-

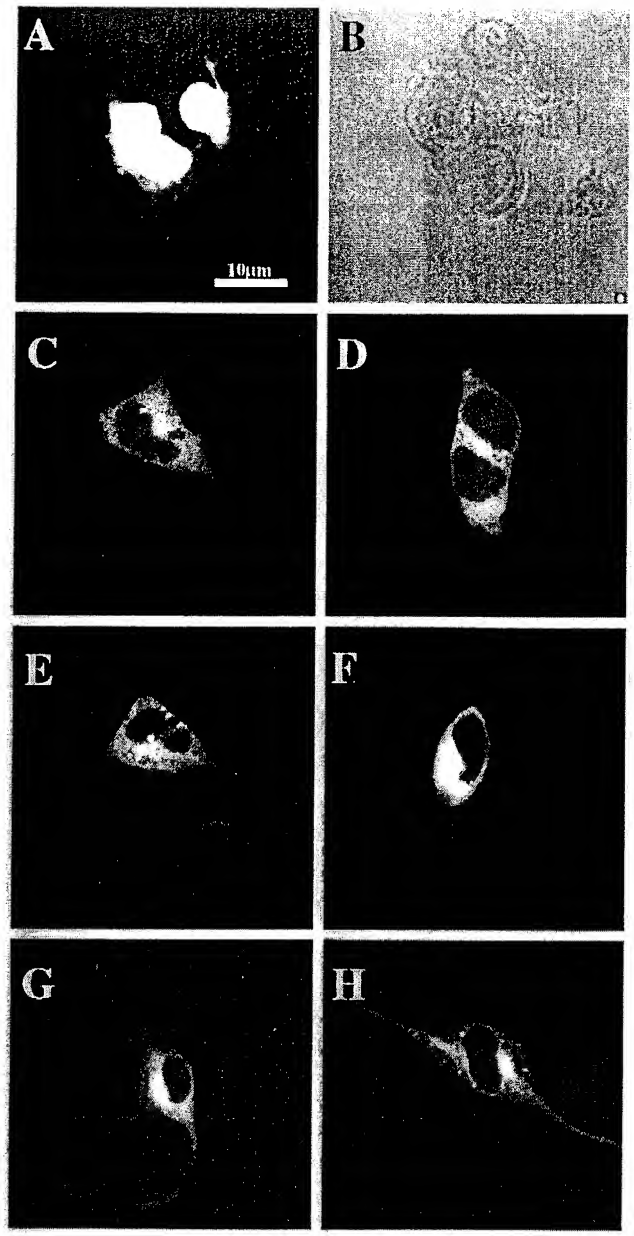


FIG. 11. Expression of hTTP-GFP fusion protein in 293 cells. 293 cells were transfected with either EGFP-N1 or hTTP-GFP fusion constructs, as described in Materials and Methods. Twenty-four hours after the transfection, cells were analyzed for GFP expression by confocal fluorescence microscopy with a fluorescein isothiocyanate filter. (A) Cells transfected with EGFP-N1 (5 μg); (B) the same field as in panel A under phase-contrast microscopy showing several nontransfected cells that were nonfluorescent; (C and D) cells transfected with CMV.hTTP.EGFP (10 μg); (E and F) cells transfected with CMV.hTTP.EGFP (5 μg); (G and H) cells transfected with H6E.EGFP (10 μg).

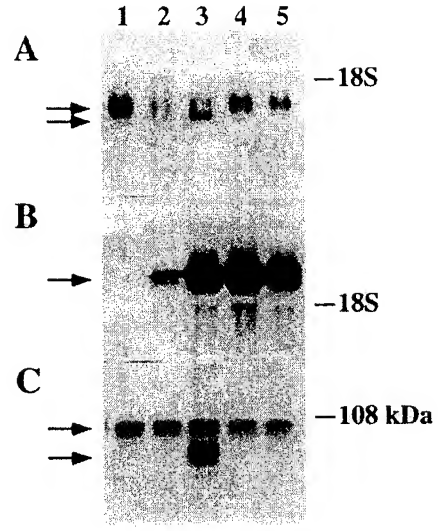


FIG. 12. Effect of GFP-TTP on TNF-α mRNA stability and binding to the ARE of mTNF-α mRNA. (A and B) CMV.mTNF-α (1 μg/plate) was cotransfected into 293 cells with 5 μg of either vector alone (lane 1), H6E.EGFP (lane 2), CMV.hTTP.EGFP (lane 3), or the zinc finger mutants (C124R, lane 4; C147R, lane 5) of CMV.hTTP.EGFP per plate. Preparation of total cellular RNA, electrophoresis, and Northern blot analysis were performed as described in Materials and Methods. Each lane was loaded with 10 μg of total RNA. The Northern blots were probed with ^{32}P-labeled mTNF-α cDNA (A) or mTTP cDNA (B). In panel A, the arrows indicate the two species of mTNF-α mRNA; the position of the 18S rRNA is also indicated. In panel B, the positions of TTP-GFP (arrow) and the 18S rRNA are indicated. (C) 293 cells were transfected with the same plasmids as described for panels A and B but without the CMV.mTNF-α. Cytosolic extracts were prepared, and 20 μg of total cytosolic protein per sample was used in UV cross-linking assays with a ^{32}P-labeled mTNF-α RNA (1281–1350) probe, followed by SDS-PAGE (10% polyacrylamide gel) and autoradiography. The top arrow indicates the position of the radiolabeled endogenous cellular protein with an M_r of ~80,000; the bottom arrow indicates the radiolabeled TTP-GFP fusion protein. The position of the 108-kDa protein standard is indicated.

ative effect on TNF-α mRNA degradation exerted by zinc finger and other mutant TTP constructs (25). Whatever the mechanism of this protective effect, it seems unlikely to be of physiological importance, given that the effect of TTP knockout in mice is to increase TNF-α mRNA stability (7). This phenomenon may nonetheless be useful in identifying potentially interacting proteins that cause the breakdown of the mRNA body.

Several proteins with TNF-α ARE-binding and cross-linking activity have been identified previously in cell extracts from macrophages and other cells (3, 4, 15, 16, 18, 20, 33, 51). In some cases, the activity was inducible by LPS (14, 26). These activities may well represent some of the ARE-binding proteins identified in our cross-linking experiments, in which endogenous proteins of ~32, 38, 66, and 82 kDa were identified in 293 cell extracts. It is unlikely, however, that TTP would have been identified by this means, since its presence in normal macrophages was barely detectable after maximal LPS stimulation with the TNF-α mRNA ARE probe and specific antibodies to TTP. However, since TTP binding to the TNF-α mRNA ARE appeared to displace some of these endogenous cellular proteins, it is conceivable that some of them exert an ARE-protective effect. In this model, TTP could cause TNF-α mRNA destabilization, at least in part, by displacing such protective proteins from the ARE.

Many questions remain to be answered about these proposed interactions, particularly concerning their specificity and physiological relevance. It is unlikely that the only mRNA species affected by TTP is that encoding TNF-α, and further experiments will be required to identify other physiologically relevant binding partners. Other future work should address the possible functions of the rapid mitogen-induced changes in TTP biosynthesis, subcellular localization, and serine phosphorylation, as well as the functions of other CCCH zinc finger protein family members. The availability of the cell-free and

transfection assays described here should help to answer these questions.

ACKNOWLEDGMENTS

We thank Bruce Beutler for the mouse TNF-α cDNA, Dori Germolec and Anton Jetten for helpful comments on the manuscript, and Jeff M. Reece for assistance with confocal microscopy.

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